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A promoter switch that can rescue a plant sigma factor mutant

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Abstract **Chloroplasts sigma factors act in concert with PEP, the bacterial-type plastid RNA polymerase. Using a sigma knockout line from *Arabidopsis thaliana*, we investigated mutant-specific changes in plastid gene expression at RNA level. One characteristic feature was the appearance of a long transcript that spans the *atpB-E* operon and extends considerably into the far-upstream region of *atpB*. This region reveals a cluster of typical promoter elements for NEP, the second (phage-type) plastid RNA polymerase. The NEP promoter cluster can help maintain RNA synthesis in situations where no functional sigma factor is available for PEP.**

Keywords: Chloroplast transcription; Plant sigma factors; Promoter cluster; RNA polymerase switch; SOS transcripts

1. Introduction

Green plant cells have three transcriptionally active compartments, i.e. the nucleus, mitochondria, and chloroplasts. The latter contain at least two types of RNA polymerases, PEP, the plastid-encoded multi-subunit enzyme, and NEP, the nucleus-encoded single-subunit enzyme [1,2]. A number of chloroplast genes are transcribed exclusively by PEP (class I), others both by PEP and NEP (class II), or by NEP alone (class III) [3,4]. NEP promoters lack the −35/−10 (PEP) promoter elements and can be subgrouped according to their architecture. The YRTA core motif is the conserved sequence element of type Ia, type Ib promoters have an additional GAA-box, and those of type II do not resemble any of the others [5,6].

The complexity of chloroplast transcription is further increased by multiple bacterial-type sigma factors for initiation of RNA synthesis by PEP [7,8]. In the model plant *Arabidopsis thaliana*, a family of six nuclear genes (*AtSig1–6*) for the plastid sigma factors SIG1–6 has been identified [9–11] and studies on the in vivo role of each individual member have become possible with sigma knockout plants [12–16]. When we looked at plastid gene expression in a novel SIG6 knockout line [17,18], we observed large transcripts normally not seen in the wildtype, which could mean that SIG6 has an unexpected post-transcriptional function in RNA maturation. Based on the data shown here, we favour another mechanism that involves promoter switching. At the center of this model is a far-upstream NEP promoter (cluster) that is used in situations when no functional sigma factor can be recruited by PEP.

2. Materials and methods

2.1. Plant material, growth conditions, RNA

The *A. thaliana sig6-2* mutant with a T-DNA insertion in exon 5 of the *AtSig6* gene was generated as part of the GABI-Kat mutation program [17]. Wildtype and *sig6-2* seedlings of *A. thaliana* (Columbia) were grown on MS agar medium containing 0.4% Gelrite and 1% sucrose (8 h short-day at 24 °C and 60 μmol m^{-2} s^{-1}) for up to 2 weeks. Young plants were then transferred onto soil for 1 or 2 weeks. The cotyledons (3–14 d) or rosette leaves (21 and 28 d) were collected, frozen and powdered in liquid nitrogen. Total RNA was prepared as decribed [18].

2.2. RT-PCR, oligonucleotides, probes

RNA (2 μg) and random primers (10 pM) were incubated with AMV-RT buffer, dNTPs (0.25 mM each), 40 U RNasin, and 30 U AMV reverse transcriptase (Promega) at 37 °C for 90 min. Hybridization probes were prepared from cDNAs that were PCR-amplified using *Taq* DNA polymerase and then cloned into pGEM-T Easy (Promega). The linearized plasmids were transcribed in vitro by phage polymerases in the presence of DIG-11-UTP (Roche).

2.3. Northern blot hybridization

RNA samples (1 μg per lane) were electrophoresed in 1.2% agarose-formaldehyde gels, blotted to positively charged nylon membranes (Roche), and hybridized with DIG-labeled RNA probes. Hybridization (68 °C for 12 h) and chemiluminescent detection were carried out as described in the Roche DIG manual.

3. Results and discussion

3.1. Large mutant-specific transcripts from the atpB region of chloroplast DNA

The *sig6-2* mutant phenotype resembles that of the wildtype, except for severe chlorophyll deficiency of the seedling (white cotyledons) [18]. To test if this stage-specific phenotype is reflected by altered expression of plastid genes (AP000423) [19], we selected a region that contains the *atpB*/*atpE* operon for the β and ε subunits of the ATPase [20] followed by the split tRNA-Val gene *trnV*(UAC) and the *ndh* genes for the subunits C, K and J of the NADP dehydrogenase complex [21].

Using an *atpB*-specific probe in Northern blot analyses, transcripts at 2.0 and 2.6 kb were detected in all wildtype samples (WT 3–28 d) (Fig. 1A, top row). The mutant (MT) always showed the smaller transcript, but the larger 2.6 kb species was present in wildtype levels only in very young (3 d) seedlings. In addition, a novel, mutant-specific, transcript 4.8 kb in size was

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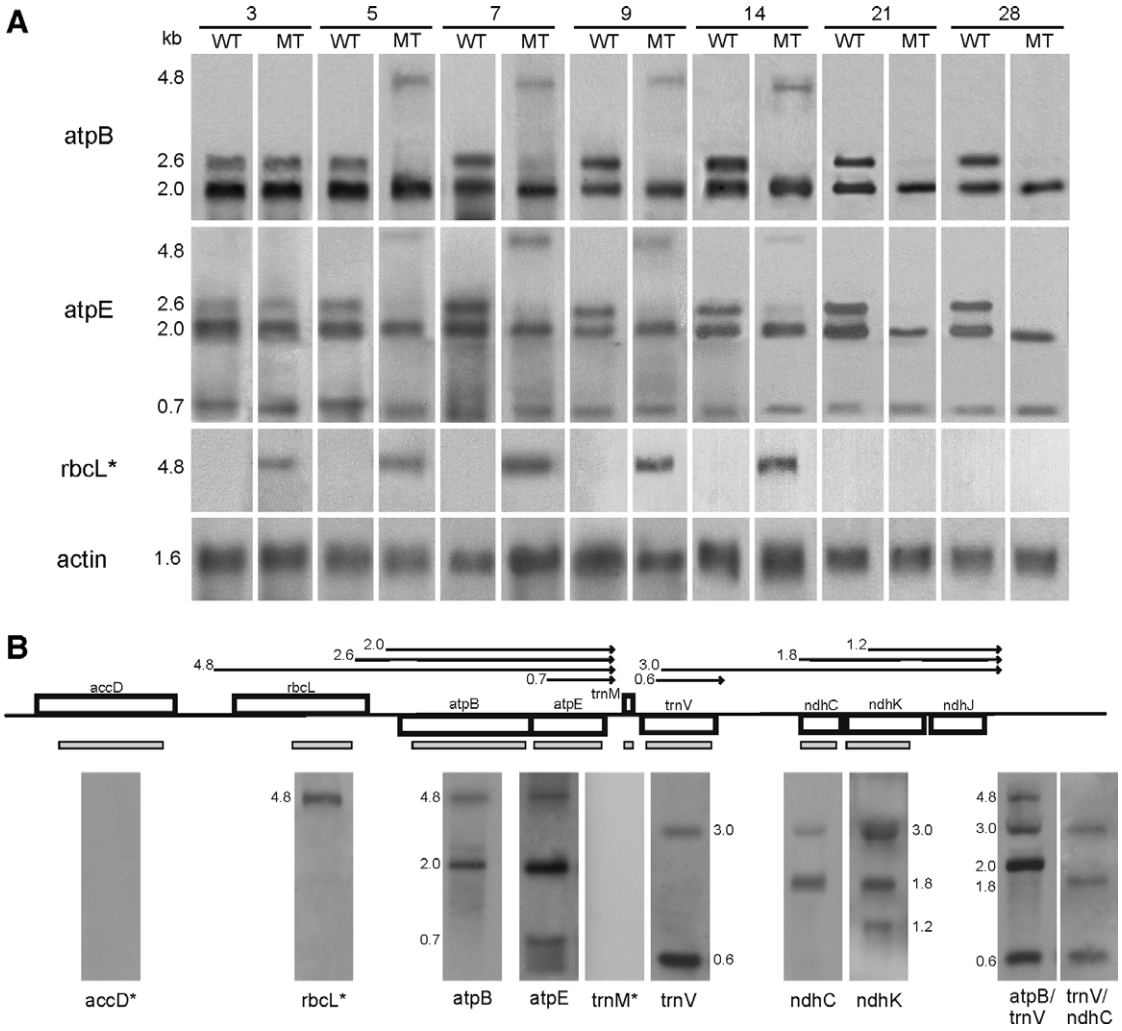

Fig. 1. Northern blot analysis of plastid gene expression in wildtype (WT) and *sig6-2* mutant (MT). (A) Total RNA (1 μg each) was hybridized with DIG-labeled RNA probes for *atpB*, *atpE*, *rbcL** (opposite strand of *rbcL*), and the nuclear control gene *actin2*. Numbers on top: age of plant material (days). Left margins: transcript sizes (kb) determined by RNA markers (Promega). (B) Assignment of *sig6-2* mutant transcripts by probes spanning distinct portions of the *accD–ndhJ* region [19]. Upper: coding regions (boxes), positions of probes (gray bars) and transcripts (arrows in 5′ to 3′ direction, sizes in kb). Lower: Transcripts after Northern blot hybridization (1 μg total RNA) from 7-day old mutant seedlings. Numbers next to lanes indicate transcript sizes (kb).

visible, which accumulated transiently during the intermediate stages (5–14 d). The same hybridization pattern was obtained by an *atpE* probe (Fig. 1A, second row), except for an additional 0.7 kb signal representing the monocistronic mRNA for the ε subunit [20].

Only the 4.8 kb transcript was seen with probe *rbcL** (for the opposite strand of the *rbcL* gene) (Fig. 1A, third row), indicating that the 5′ end of this large RNA species is far-upstream from *atpB*. Interestingly, this probe detected the large transcript already at the 3 day-stage. This apparent discrepancy compared to the results obtained with *atpB*/*E* probes may be due to competition hybridization by the 2.0 and 2.6 kb transcripts detected by the latter probes (see drawing Fig. 1B). Hence, although the *rbcL** experiments support the conclusion based on atpB/E hybridization that the 4.8 kb transcript has transient kinetics with peak levels at day 7, the accumulation seems to start even earlier than expected.

Of the additional probes on either side of *atpB* (Fig. 1B), probe *accD** (for the opposite strand of *accD*) on the left did not detect any signal, whereas probes *rbcL**, *atpB*, and *atpE* all showed the 4.8 kb species. Transcripts of 0.6 and 3.0 kb but not 4.8 kb were detected by the *trnV* probe, and none at all by the *trnM** probe. The double-probe *atpB*/*trnV* as well as probes *ndhC*, *ndhK* and *trnV*/*ndhC* together confirmed the 4.8 and 3.0 kb bands as distinct transcripts. These hybridization data are consistent with the RNA 3′ end of the mutant-specific 4.8 kb *atpB*/*E* transcript between *atpE* and *trnM*. Considering the short (137 bp) distance between the *atpE* coding region and *trnM* on the opposite strand [19] (Fig. 1B) as well as the transcript size, the RNA 5′ end can be placed 4.8 kb to the left between *rbcL* and *accD* on the opposite strand. It should be noted that the *atpE* probe covers almost the entire *atpE* coding region and therefore would give a signal even if the 4.8 kb transcript would end within this region. In this scenario, however, the 5′ end would be within the *accD* region (opposite strand), which is obviously not the case because of the absence of a hybridization signal with the *accD* *probe (Fig. 1B).

3.2. Motif search upstream of atpB detects putative NEP promoter cluster

With a focus on *atpB*/*E*, we tried to correlate the multiple transcripts with putative PEP or NEP promoters [2,8]. In tobacco, 4 PEP (NtP*atpB-255*, −488, −502, and −611) and 1 NEP promoter (P*atpB-290*) are known in front of *atpB* [22,23]. In *A. thaliana*, a single PEP promoter named P*atpB-515*/*-520* was recently mapped [24] (K. Liere, personal communication).

The 2.6 kb *atpB*/*E* RNA (Fig. 2) was always abundant in wildtype but highly diminished in the mutant, suggesting that it might be a (sigma-dependent) PEP transcription product from P*atpB-515*/*520* (Fig. 2B). The 2.0 kb RNA was always present at wildtype amounts in the mutant, consistent with SIG6-independent synthesis of this species by NEP. Indeed, AtP*atpB-321* in front of *atpB* shows sequence similarity with NtP*atpB-290*, the functionally defined NEP promoter from tobacco (Fig. 2A, top) [6].

The region in front of the 4.8 kb transcript did not show conserved −35/−10 (PEP) motifs but seven closely-spaced GAA/YRTA elements of NEP Ib promoters [6] 2473–2707 nt from the *atpB* translation start site (Fig. 2A, bottom). This

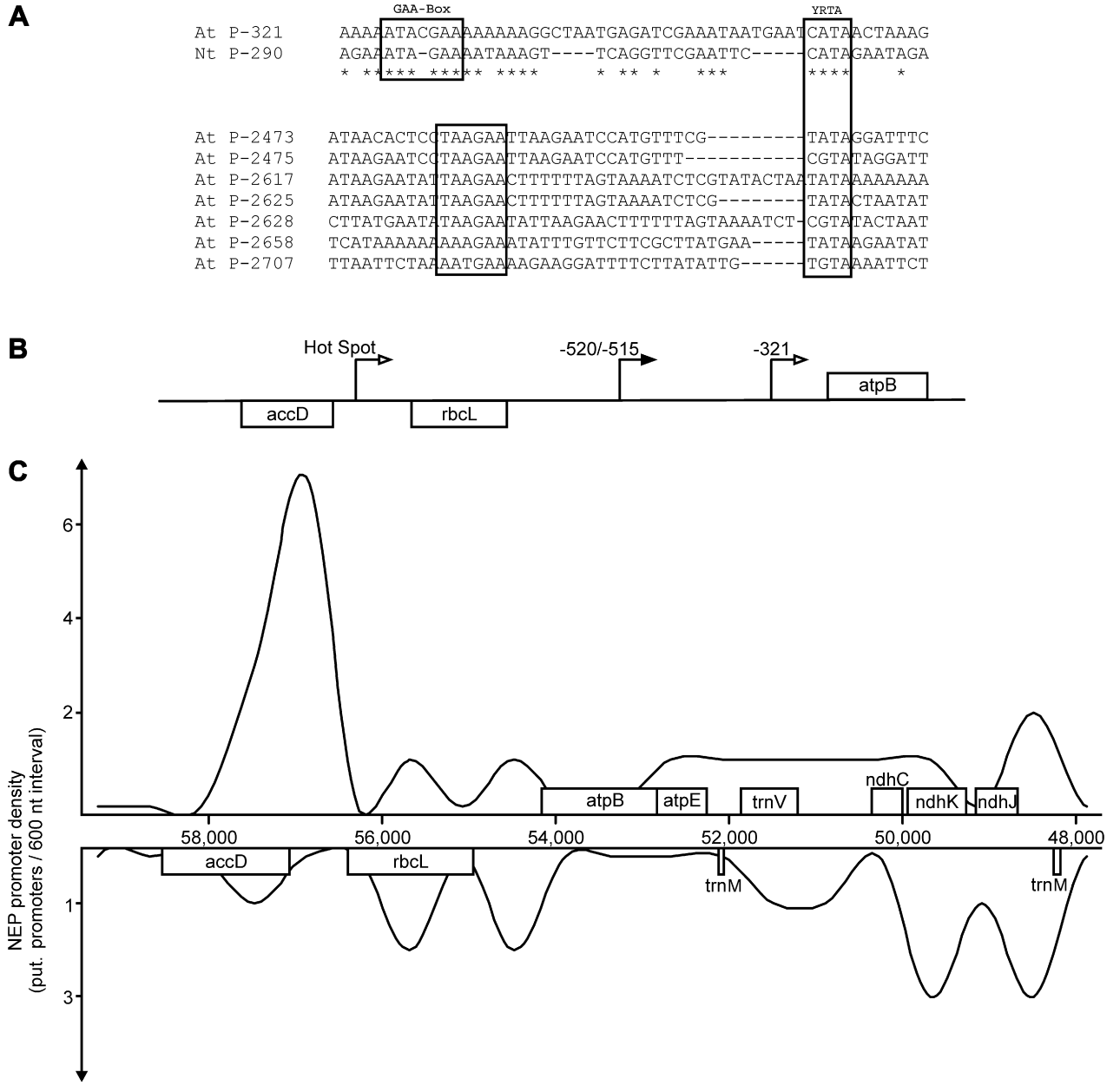

Fig. 2. Promoter search upstream of the *atpB* gene from *A. thaliana*. (A) CLUSTAL W alignments of 5′ regions from *A. thaliana* (At) and *N. tabacum* (Nt). Putative NEP promoter elements are boxed, including those far-upstream of *atpB* (hot spot). (B) Mapped PEP (black [24]) and putative NEP (white) promoters in front of *atpB*. Numbers: distance (bp) from translation start site. (C) A*ccD* to *trnM* region (49 981–60 180 on the map [19]). Frequency of putative NEP promoters was counted in 600-bp intervals and the results were plotted by using the line interpolation option of Excel®.

'hot spot' (Fig. 2B) in the far-upstream 5′ region of *atpB* is readily discerned, with a peak density exceeding the average value of the entire *accD–ndhJ* region by more than a factor 5 (Fig. 2C).

To see if other plant species have similar clusters in this region, sequence alignments were carried out, showing enhanced density of NEP promoters in rice, tobacco and maize. The distance between hot spot and *atpB* coding region was in the range of 2.3 kb (rice) to 2.6 kb (tobacco and maize). Furthermore, the inner circle of the DNA molecule reveals putative NEP promoter clusters in 3 other intergenic regions (*rps12*, *trnE* and *trnQ*) (not shown).

3.3. High-molecular transcripts in PEP-deficient mutants

High-molecular plastid transcripts are noticeable also in *A. thaliana* knockouts for other components of the PEP transcription machinery (see e.g. [24]), which was interpreted as a defect in RNA maturation [25,26]. Existing evidence suggests coupling of chloroplast transcription with the stability and turnover of RNAs [27,28]. Although we did not exclude posttranscriptional mechanisms here, we emphasize an explanation based on differential promoter usage. This is borne out by the findings that the 4.8 kb RNA is preceded by clustered NEP promoter elements in the far-upstream region of *atpB* (Fig. 3), and similar clusters exist in homologous positions in other plants, and in other regions of the *A. thaliana* plastom.

3.4. Promoter switch model for stage-specific changes in plastid gene expression

In wildtype (Fig. 3, top) the two transcripts of 2.0 and 2.6 kb were always detected, which may mean that both the PEP (P*atpB-515*/*-520*) [24] and the proximal NEP (P*atpB-321*) promoters are used throughout development. In contrast, three different temporal modes of transcription can be envisaged in the mutant (Fig. 3, bottom): (i) In 3-day-old mutant seedlings and probably earlier (embryo stage) the situation still resembles that in wildtype, suggesting that at least one other sigma factor may be able to substitute for AtSIG6 at this stage. This could be any of AtSIG1-5, formally termed eSIG (embryonal sigma factor). (ii) The almost complete disappearance of the 2.6 kb (PEP) transcript by day 5 suggests a promoter-specific requirement for AtSIG6, which no longer can be efficiently substituted by other factors at this stage. The far-upstream promoter cluster is now used by NEP, which counteracts the loss of SIG6/PEP-dependent transcription and ensures RNA levels beyond those driven by the proximal NEP promoter. This SOS function may be required for rescue of the transcription system and transition from the chlorophyll-defective seedling to the healthy plant. (iii) Disappearance of the transient 4.8 kb transcript may reflect loss of the SOS function at a time when the 2.0 kb transcript from the proximal NEP promoter alone is sufficient.

Plastid promoter switching, in principle, can involve both the PEP and NEP type of RNA polymerase and associated

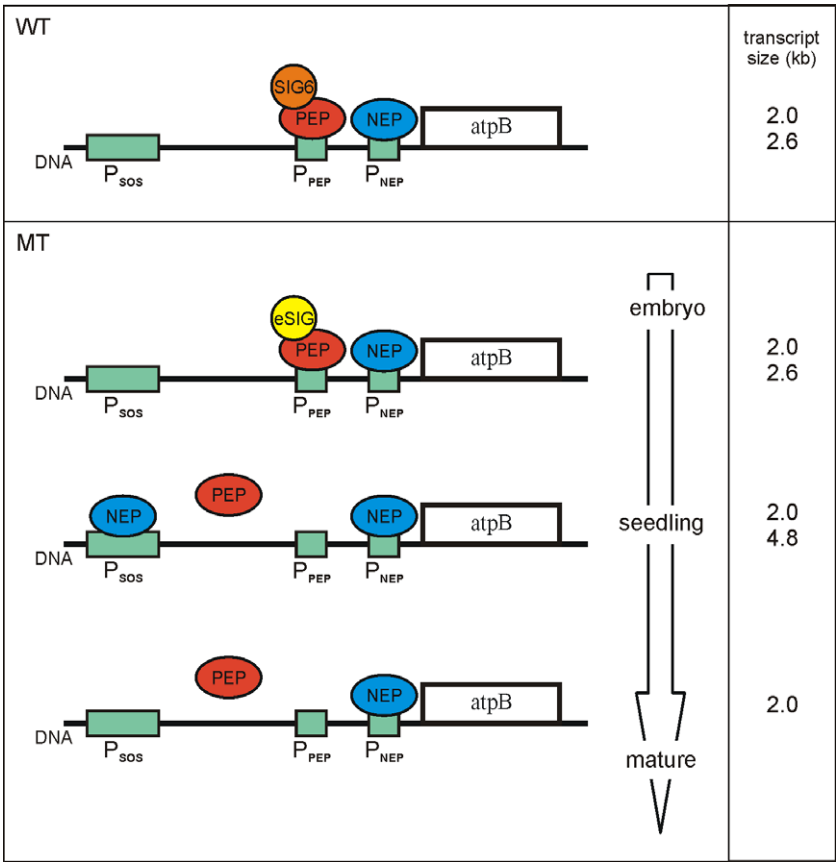

Fig. 3. Model suggesting promoter switch regulation of RNA size distribution during development. Top: wildtype (WT). Both the PEP and proximal NEP promoter are used. Bottom: mutant (MT). Promoter usage differs from that in WT, including developmental stage-specific switch. Colors: green (promoters), red (PEP), blue (NEP), orange (SIG6), yellow ('embryonal' sigma factor eSIG, which could be any of SIG1-5). RNA sizes (kb) are given in the right margin.

factors. The initial recruitment of NEP to the far-upstream promoter cluster may be facilitated by the absence of a functional SIG6/PEP-promoter complex further downstream. Following the SOS activity, the subsequent release of NEP from the upstream promoter could be mediated by a regulator such as $tRNA^{Glu}$ [29].

Why are both the proximal and far-upstream NEP promoters necessary in the mutant? The 2.0 kb transcript driven by the proximal NEP promoter (P*atpB*-321) lacks 5′UTR sequences that are provided by the 2.6 kb (PEP-driven) species in wildtype. The mutant-specific 4.8 kb transcript from the far-upstream promoter cluster contains such UTR sequences, which might be required for high-level translation and/or other regulatory purposes after appropriate RNA processing [28,30].

It should be kept in mind that the NEP promoters were assigned here on the basis of their location in front of the corresponding (4.8 and 2.0 kb) transcripts and their similarity to previously mapped plastid promoters [6,31] (Fig. 2). In plant mitochondria, significant deviation from the consensus architecture has recently been observed for a number of NEP promoters [32], and increasing evidence has accumulated for the existence of two different NEP enzymes in plastids [33,34]. In an extension of the model (Fig. 3) it therefore could be envisaged that one NEP enzyme is responsible for the "constitutive" 2.0 kb transcript, whereas a second NEP is involved in the SOS activation at the far-upstream promoter cluster for the transient 4.8 kb transcript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.010.

References

[1] Hedtke, B., Börner, T. and Weihe, A. (1997) Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. Science. 277, 809–811.
[2] Maliga, P. (1998) Two plastid RNA polymerases of higher plants: an evolving story. Trends Plant Sci. 3, 4–6.
[3] Hajdukiewicz, P.T., Allison, L.A. and Maliga, P. (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. EMBO J. 16, 4041–4048.
[4] Kapoor, S., Suzuki, J.Y. and Sugiura, M. (1997) Identification and functional significance of a new class of non-consensus-type plasmid promoters. Plant J. 11, 327–338.
[5] Liere, K. and Maliga, P. (1999) In vitro characterization of the tobacco *rpoB* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters. EMBO J. 18, 249–257.
[6] Liere, K. and Maliga, P. (2001) Plastid RNA polymerases in higher plants in: Regulation of Photosynthesis (Aro, E.-M. and Andersson, B., Eds.), pp. 29–49, Kluwer, Dordrecht, The Netherlands.
[7] Hess, W.R. and Börner, T. (1999) Organellar RNA polymerases of higher plants. Int. Rev. Cytol. 190, 1–59.
[8] Shiina, T., Tsunoyama, Y., Nakahira, Y. and Khan, M.S. (2005) Plastid RNA polymerases, promoters, and transcription regulators in higher plants. Int. Rev. Cytol. 244, 1–68.
[9] Isono, K., Shimizu, M., Yoshimoto, K., Niwa, Y., Sahto, K., Yokota, A. and Kobayashi, H. (1997) Leaf-specifically expressed genes for polypeptides destined for chloroplasts with domains of sigma70 factors of bacterial RNA polymerases in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 94, 14948–14953.
[10] Tanaka, K., Tozawa, Y., Mochizuki, N., Shinozaki, K., Nagatani, A., Wakasa, K. and Takahashi, H. (1997) Characterization of three cDNA species encoding plastid RNA polymerase sigma factors in *Arabidopsis thaliana*: evidence for the sigma factor heterogenity in higher plant plastids. FEBS Lett. 413, 309–313.
[11] Hakimi, M.-A., Privat, I., Valay, J.-G. and Lerbs-Mache, S. (2000) Evolutionary conservation of C-terminal domains of primary $sigma^{70}$-type transcription factors between plants and bacteria. J. Biol. Chem. 275, 9215–9221.
[12] Fujiwara, M., Nagashima, A., Kanamaru, K., Tanaka, K. and Takahashi, H. (2000) Three new nuclear genes, *sigD*, *sigE* and *sigF*, encoding putative plastid RNA polymerase σ factors in *Arabidopsis thaliana*. FEBS Lett. 481, 47–52.
[13] Hanaoka, M., Kanamaru, K., Takahashi, H. and Tanaka, K. (2003) Molecular genetic analysis of chloroplast gene promoters dependent on SIG2, a nucleus-encoded sigma factor for the plastid-encoded RNA polymerase, in *Arabidopsis thaliana*. Nucleic Acids Res. 31, 7090–7098.
[14] Nagashima, A., Hanaoka, M., Motohashi, R., Seki, M., Shinozaki, K., Kanamaru, K., Takahashi, H. and Tanaka, K. (2004) DNA microarray analysis of plastid gene expression in an *Arabidopsis* mutant deficient in a plastid transcription factor, SIG2. Biosci. Biotechnol. Biochem. 68, 694–704.
[15] Ishizaki, Y., Tsunoyama, Y., Hatano, K., Ando, K., Kato, K., Shinmyo, A., Kobori, M., Takeba, G., Nakahira, Y. and Shiina, T. (2005) A nuclear-encoded sigma factor, *Arabidopsis* SIG6, recognizes sigma-70 type chloroplast promoters and regulates early chloroplast development in cotyledons. Plant J. 42, 133–144.
[16] Favory, J.-J., Kobayashi, M., Tanaka, K., Peltier, G., Kreis, M., Valay, J.-G. and Lerbs-Mache, S. (2005) Specific function of a plastid sigma factor for *ndhF* gene transcription. Nucleic Acids Res. 33, 5991–5999.
[17] Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K. and Weisshaar, B. (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. 53, 247–259.
[18] Loschelder, H., Schweer, J., Link, B. and Link, G. (2006) Dual temporal role of plastid sigma factor 6 in *Arabidopsis* development. Plant Physiol. 142, 642–650.
[19] Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E. and Tabata, S. (1999) Complete structure of the chloroplast genome of *Arabidopsis thaliana*. DNA Res. 6, 283–290.
[20] Westhoff, P., Nelson, N., Bünemann, H. and Herrmann, R.G. (1981) Localization of genes for coupling factor subunits on the spinach plastid chromosome. Curr. Genet. 4, 109–120.
[21] Sugita, M. and Sugiura, M. (1996) Regulation of gene expression in chloroplasts of higher plants. Plant Mol. Biol. 32, 315–326.
[22] Xie, G.L. and Allison, L.A. (2002) Sequences upstream of the YRTA core region are essential for the transcription of the tobacco *atpB* NEP promoter in chloroplast in vivo. Curr. Genet. 41, 176–182.
[23] Liere, K., Kaden, D., Maliga, P. and Börner, T. (2004) Overexpression of phage-type RNA polymerase *RpoTp* in tobacco demonstrates its role in chloroplast transcription by recognizing a distinct promoter type. Nucleic Acids Res. 32, 1159–1165.
[24] Pfalz, J., Liere, K., Kandlbinder, A., Dietz, K.J. and Oelmüller, R. (2006) pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. Plant Cell 18, 176–197.
[25] Hess, W.R., Hoch, B., Zeltz, P., Hübschmann, T., Kössel, H. and Börner, T. (1994) Inefficient *rpl12* splicing in barley mutants with ribosome-deficient plastids. Plant Cell 6, 1455–1465.
[26] Allison, L.A., Simon, L.D. and Maliga, P. (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. EMBO J. 15, 2802–2809.
[27] Legen, L., Kemp, S., Krause, K., Profanter, B., Herrmann, R.G. and Maier, R.M. (2002) Comparative analysis of plastid

transcription profiles of entire plastid chromosomes from tobacco attributed to wild-type and PEP-deficient transcription machineries. Plant J. 31, 171–188.
[28] Cahoon, A.B., Harris, F.M. and Stern, D.B. (2004) Analysis of developing maize plastids reveals two mRNA stability classes correlating with RNA polymerase type. EMBO Rep. 5, 801–806.
[29] Hanaoka, M., Kanamaru, K., Fujiwara, M., Takahashi, H. and Tanaka, K. (2005) Glutamyl-tRNA mediates a switch in RNA polymerase use during chloroplast biogenesis. EMBO Rep. 6, 545–550.
[30] Herrin, D.L. and Nickelsen, J. (2004) Chloroplast RNA processing and stability. Photosynth. Res. 82, 301–314.
[31] Weihe, A. and Börner, T. (1999) Transcription and the architecture of promoters in chloroplasts. Trends Plant Sci. 4, 169–170.
[32] Kühn, K., Weihe, A. and Börner, T. (2005) Multiple promoters are a common feature of mitochondrial genes in *Arabidopsis*. Nucleic Acids Res. 33, 337–346.
[33] Azevedo, J., Courtois, F. and Lerbs-Mache, S. (2006) Subplastidial localization of two different phage-type RNA polymerases in spinach chloroplasts. Nucleic Acids Res. 33, 436–444.
[34] Emanuel, C., von Groll, U., Müller, M., Börner, T. and Weihe, A. (2006) Development- and tissue-specific expression of the *RpoT* gene family of *Arabidopsis* encoding mitochondrial and plastid RNA polymerases. Planta 223, 998–1009.